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Toxicity of trinitrotoluene to sheepshead minnows in water exposures

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ABSTRACT

Lethal effects of trinitrotoluene (TNT) to juvenile sheepshead minnows (JSHM) (*Cyprinodon variegatus*) were assessed in ten-day water exposures. Ten-day median lethal concentrations (LC50s) were 2.3 and 2.5 mg L^{-1}, the 10-d median lethal residue value (LR50) was 26.1 μmol kg^{-1} wet weight (ww), and bioconcentration factors (BCFs) ranged from 0.7 to 2.4 L kg^{-1}. The lethal effects of TNT and its transformation products 2-aminodinitrotoluene (2-ADNT), 2,4-diaminonitrotoluene (2,4-DANT) and trinitrobenzene (TNB) to JSHM were compared in 5-d static-renewal exposures. Nitroreduction decreased the toxicity of TNT to SHM, as the 5-d LC50 for 2-ADNT was 8.6 mg L^{-1} and the lowest lethal concentration of 2,4-DANT was 50.3 mg L^{-1}. TNB (5-d LC50 = 1.2 mg L^{-1}) was more toxic than TNT to SHM. The 5-d LR50s were 4.3 mg kg^{-1} ww (20.4 μmol kg^{-1}) for SumTNT (TNT exposure) and 54.2 mg kg^{-1} ww (275.3 μmol kg^{-1}) for 2-ADNT and significant mortality occurred at 47.4 mg kg^{-1} ww (283.6 μmol kg^{-1}). The range of BCF values was from 1.8 to 2.4, 5.6 to 8.0, and 0.6 to 0.9 L kg^{-1} for TNT, 2-ADNT, and 2,4-DANT, respectively.

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1. Introduction

Explosives have been of environmental concern at military sites throughout the world due to release associated with manufacture, handling, and disposal operations. Unexploded ordnance (UXO) and dumped ammunition are present in aquatic environments (Darrach et al., 1998; Dave, 2003; Ek et al., 2006). The ordnance compounds most commonly used, and therefore, most likely to be of potential concern at aquatic sites, include 2,4,6-tritnitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (royal demolition explosive [RDX]), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (high-melting explosive [HMX]). As the ordnance items may leak munitions compounds contained within them over time, they represent a potential source of contamination and potential risk in localized areas around individual projectiles (Ek et al., 2006). Low concentrations of some explosive compounds have been measured in marine sediment (Darrach et al., 1998; Rodacy et al., 2000; Dave, 2003; Ek et al., 2006) and exposure to sediment surrounding cleaved TNT-filled artillery shells placed at the sea bottom resulted in decreased survival of a sensitive benthic copepod (Ek et al., 2006). Only trace concentrations (parts per billion or less) of explosives are expected in water surrounding ordnance cracked open on the ocean floor (Darrach et al., 1998; Rodacy et al., 2000; Dave, 2003).

Although contamination of surface freshwaters, ground waters, soils, and sediments with explosives have been documented (Talmage et al., 1999; Monteil-Rivera et al., 2009), relatively little is known about the degree of contamination in coastal marine environments and the potential effects of explosives to marine organisms. Several studies reported the toxicity of TNT to freshwater fish including rainbow trout (*Oncorhynchus mykiss*), fathead minnows (*Pimephales promelas*), channel catfish (*Ictalurus punctatus*), and bluegill sunfish (*Lepomis macrochirus*) (Pederson 1970; Nay et al., 1974; Smock et al., 1976; Liu et al., 1983a; Bailey and Spanggord 1983; Bailey et al., 1985; Yoo et al., 2006). The toxicity metrics reported in those studies were primarily LC50 values from 48-h and 96-h exposures that ranged from 0.8 to 3.7 mg L^{-1} for the various fish species. The single published study of the toxicity of TNT to marine fish reported a 48-h LC50 value of 7.6 mg L^{-1} for newly hatched redfish (*Poecilia reticulata*) larvae (Nipper et al., 2001).

Once released into the aquatic environment, TNT typically undergoes rapid transformation (Monteil-Rivera et al., 2009). Comparison of the toxicity of TNT and its reduced transformation products to a variety of aquatic invertebrates revealed that lethal concentrations of TNT were remarkably similar for species belonging to different taxonomic groups. However, aminated TNT transformation products (ADNTs and DANTs) were typically less toxic than the parent compound and their toxicity varied much more broadly across species (Won et al., 1976; Griest et al., 1998; Steevens et al., 2002; Lotufo and Farrar 2005). The toxicity of TNT transformation products to fish, reviewed in Nipper et al. (2009), has not been reported in the current scientific literature.

In general, little information exists regarding the potential for major explosives to bioaccumulate in fish tissues. A bioconcentration factor (BCF) is the ratio of the concentration of a chemical

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in an aquatic organism to that in the surrounding water and is the most frequently used indicator of a compound's propensity to bioconcentrate in aquatic organisms. Previously, the range of bioconcentration factors for fish was determined to be low for TNT (0.8–9.7 L kg^{-1}, Ownby et al., 2005; Lotufo and Lydy 2005; Yoo et al., 2006), 2-ADNT (13.1 L kg^{-1}, Lotufo and Lydy 2005), and 2,4-DANT (0.5 L kg^{-1}, Lotufo and Lydy 2005). TNT tissue concentrations in fish associated with significant toxicity (critical body burden) have not been reported in the available literature.

The objective of this study was to derive toxicological information to assess the potential toxicity of explosives compounds to marine fish. TNT toxicity and bioaccumulation data were collected using juvenile sheepshead minnows (JSHM, *Cyprinodon variegatus*). Five- and ten-day exposures to increasing concentrations of 2,4,6-trinitrotoluene (TNT), 1,3,5-trinitrobenzene (TNB), 2-aminodinitrotoluene (2-ADNT), and 2,4 diaminonitrotoluene (2,4-DANT) dissolved in water were used to assess survival, bioconcentration, and to develop relationships between body burden and observed effects. In addition, for TNT, static-renewal and flow-through exposure scenarios were compared.

2. Materials and methods

2.1. Experimental organisms

Laboratory-cultured JSHM were purchased from Aquatic BioSystems (Fort Collins, CO) and shipped overnight to the U.S. Army Corp of Engineer's Research and Development Center (Vicksburg, MS) approximately one week prior to experiment initiation.

Sheepshead minnows are found in estuaries along the southeastern and eastern coast of the United States and are widely used in routine toxicity testing of whole effluent and receiving waters (ASTM 1999). Experimental fish used in this study were treated humanely, and their use did not violate regulations as per the Department of Defense document, "The Care and Use of Laboratory Animals in DOD Programs" (Army Regulation 40-33; SECNAVINST 3900.38C).

2.2. Chemicals

Chemicals used in the exposures included 2,4,6-trinitrotoluene (TNT), 1,3,5-trinitrobenzene (TNB), 2-aminodinitrotoluene (2-ADNT), and 2,4 diaminonitrotoluene (2,4-DANT). TNT was obtained from Chem Service (West Chester, PA), TNB was obtained from Supelco (Bellefonte, PA). 2-ADNT and 2,4-DANT were obtained from SRI International (Menlo Park, CA). The manufacturer-reported purity was >98% for all compounds.

2.3. Static-renewal TNT toxicity experiment

Static-renewal exposures with daily renewal of 90% of the exposure water were conducted to determine the toxicity of TNT to JSHM (15–30 mg wet wt., approximately 5 weeks old). Fish were acclimated for 3 d in reconstituted seawater (RSW, Crystal Sea®, Marine Enterprises International, Essex, MD). Juvenile fish were exposed to five TNT-fortified treatments and to a control treatment. The appropriate amount of TNT dissolved in acetone was added to 20 psu RSW to create the highest desired concentration (4 mg L^{-1}). The reported saturation limit for TNT is 88.5 mg L^{-1} at 20 °C (Brannon and Pennington, 2002). Acetone was dissolved in 20 psu RSW to create dilution water used to prepare 4 additional TNT treatments. The concentration of acetone in the control and all TNT treatments was 1 ml L^{-1}. The TNT-spiked water solutions and dilution water were mixed in different proportions to create concentrations ranging from 0.5 to 4.0 mg L^{-1} (Table 1).

Five replicates were used per treatment. Each replicate consisted of ten fish exposed to aqueous TNT solutions in 600 ml glass beakers. Test beakers were held in a recirculating water bath system at 23 °C under yellow fluorescent light ($\lambda > 500$ nm) to minimize photodegradation of the explosives. Water quality (pH, temperature, dissolved oxygen, salinity) was measured at exposure initiation and termination. Gentle aeration using a bubbler was necessary to maintain adequate and constant oxygen concentration. TNT degraded to its transformation products under the conditions of the experiment, resulting in exposure solutions comprising a mixture of TNT and those transformation products. Such mixtures are hereafter referred to as SumTNT.

Fish were fed *Artemia salina* nauplii daily according to USEPA (2002). Dead fish were removed before each water exchange. Exposure water was sampled from one replicate beaker randomly selected before and after each renewal for chemical concentration determination. At the end of the 10-d exposure period, the experiment was terminated. All surviving fish from each replicate were removed and stored at −20 °C for body residue analysis.

2.4. Flow-through TNT toxicity experiment

The toxicity of TNT to JSHM was investigated using a diluter board capable of delivering a precise amount of exposure water to experimental chambers every hour in an attempt to maintain constant exposure concentrations. Following laboratory acclimation (7 days), fish, approximately 4–5 weeks old (15–20 mg wet wt.), were exposed to five concentrations of TNT, in addition to a solvent control. A 4 mg L^{-1} solution and a solvent control solution were prepared using a concentration of 0.8 ml L^{-1} acetone and 20 psu RSW. Those solutions were held in separate 380-L tanks. The TNT solution and the solvent control solution were mixed in the appropriate proportions and delivered to the exposure chambers by the diluter board apparatus. The measured concentrations of the diluted solutions at experiment initiation were 0.337, 0.635, 1.039, 1.975, and 4.000 mg L^{-1}, which corresponded to 4.4%, 8.4%, 15.9%, 26.0%, 49.4% and 100.0% of the highest concentration. The concentrations of TNT in the holding tanks were not measured at later time points.

Each treatment had four replicates, the maximum number handled by the diluter board. Each replicate consisted of 12 fish exposed in 2.5-L glass square tanks ($16 \times 13 \times 13$ cm^3). Fish were fed *A. salina* nauplii daily according to USEPA (2002). Approximately 190 ml of exposure water was delivered to each exposure chamber 48 times every 24 h (twice every hour). Therefore, each chamber received approximately 400% of its holding capacity (4 full volume additions) every 24 h. Water exited the chambers via ports located 1.7 cm from the top lip. Test tanks were held in a recirculating water bath system at 23 °C under yellow fluorescent light ($\lambda > 500$ nm) to minimize photodegradation of the explosives. Water quality (pH, temperature, dissolved oxygen, salinity) was measured at the exposure initiation and termination. Dead fish were removed daily, counted and discarded. For each treatment, exposure water samples were taken twice daily from two randomly selected chambers for chemical concentration determination. At the end of the 10-d exposure period, the experiment was terminated and all surviving fish from each replicate were counted. Because the primary objective of this experiment was to compare temporal trends of exposure concentrations and their relationship to toxicity using static-renewal and flow-through systems, exposed fish were not used for body residue determination

2.5. Nitroaromatic compound comparative toxicity experiment

Aqueous exposures were conducted to compare the toxicity of TNT and the TNT transformation products TNB, 2-ADNT, and 2,4-DANT to JSHM. The

Table 1
Static-renewal TNT toxicity experiment. Target and mean concentrations of TNT, mean percent of transformation, percent change in concentration of exposure water after the 24-h period following water exchange, and day 4 and day 10 mean percent of fish survival. Numbers in parentheses are ±1 standard deviation.

Target concentration (mg L^{-1})	Measured concentration (mg L^{-1}) TNT+4-ADNT	Twenty-four-h aged water		Mean survival (%)	
		% 4-ADNT	% decrease TNT+4-ADNT	Day 4	Day 10
0	0	0	0	100.0 (0.0)	100.0 (0.0)
0.5	0.31 (0.06)	56.9 (26.8)	69.2 (16.9)	98.0 (4.5)	98.0 (4.5)
1.0	0.69 (0.11)	28.2 (30.4)	54.0 (27.1)	98.0 (4.5)	98.0 (4.5)
2.0	1.52 (0.16)	7.3 (5.1)	43.6 (19.9)	94.0 (8.9)	88.0 (11.0)
3.0	2.38 (0.50)	4.7 (3.6)	39.7 (23.0)	**60.0 (15.8)**	**54.0 (15.2)**
4.0	3.14 (0.72)	6.0 (9.1)	44.2 (28.4)	**12.0 (8.4)**	**12.0 (8.4)**

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transformation 4-ADNT was not investigated because a source for obtaining the necessary mass of that compound was not identified at the time of the investigation. Exposures were conducted using 4-week-old juvenile fish (15–25 mg). Following a 3-d laboratory acclimation period, fish were exposed for 5 days to 5 concentrations of TNT, 2-ADNT and TNB solutions and 4 concentrations of 2,4-DANT solutions, in addition to a control treatment. Exposure water preparation and fish exposure were conducted as described for the static-renewal TNT toxicity experiment except for the use of 300 ml beakers as exposure vessels. A smaller exposure vessel was because a limited supply of 2-ADNT and 2,4-DANT was available to prepare exposure water for those chemicals. The 5-d exposure period was considered adequate for comparing the toxicity of TNT and some of its transformation products because virtually no additional fish mortality occurred between days 5 and 10 in the TNT static-renewal exposure.

2.6. Chemical analysis

Overlying water was sampled directly from the exposure tanks. Water samples were assayed for analytes as described below. Live fish were collected from each replicate upon termination of the explosive compound comparative toxicity experiment, pooled, and then frozen. Whole fish were extracted for chemical analysis using the following method, developed for small (50–200 mg) tissue samples. Whole fish, stored in pre-weighed 1.5 ml bead beater sample vials, were first thawed and sliced within the vials using a stainless steel scalpel. Then 100 mg of 1 mm glass beads (Biospec Products, Barttlesville, OK) and 0.2–0.75 ml of HPLC grade acetonitrile were added to the vials (volume was dependent upon the wet weight of sample; i.e. <100 mg samples received 0.2 ml, 100–200 mg samples received 0.3 ml, 200–300 mg received 0.45 ml and >300 mg received 0.75 ml). Samples were homogenized on a mini-bead beater (Biospec Products, Barttlesville, OK) for 200 s at a speed setting of 4200 oscillations min^{-1} and immediately placed on ice to cool. Samples were sonicated for 1 h (Branson 3200, Branson Ultrasonics Corporation, Danbury, CT) at 18 °C in a water bath (Neslab RTE-111, Neslab Instruments, Inc., Newington, NH), followed by centrifugation for 10 min at 7500g (10,000 rpm) and 4 °C. Supernatant (0.15 ml) was removed into a syringe (Nalge Nunc International, Rochester, NY; Norm-Ject (5 ml) Tuttlingen, Germany), which was followed by attachment of a small 0.45 μm PTFE filter to the syringe by a luer-lock. Cold 1% $CaCl_2$ (0.15 ml) was added to the syringe and the sample was filtered into Eppendorf tubes and transferred into amber HPLC vials using a glass Pasteur pipette. Samples were stored at 4 °C under dark conditions for HPLC analysis.

Aqueous samples and solvent extracts were separated and quantified by high performance liquid chromatography (HPLC) following the U.S. Environmental Protection Agency SW-486 Method 8330. The quantified compounds included TNT, TNB, the TNB transformation products dinitroanilines (DNAs), by concurrent detection of 2,4-DNA and 3,4-DNA isomers, and the TNT transformation products 2-ADNT, 4-ADNT, and DANTs (concurrent detection of 2,4-DANT and 2,6-DANT isomers). Analyses were conducted with an Agilent 1100 Series HPLC (Palo Alto, CA) equipped with a Supelco RP-Amide C-16 column and a photo diode array detector. Sample injection volume was 100 μl with a flow rate of 1 ml per minute and column temperature of 45 °C using an isocratic mobile phase consisting of 55% methanol and 45% water. Absorbance was measured at 230 and 254 nM. Peak identification was based on retention time with spectral analysis confirmation. The laboratory reporting limit for all analytes was ~0.1 mg L^{-1} for water samples, and ~1 mg kg^{-1} wet weight (ww) for tissue samples. Recoveries ranged from 90% to 98%.

2.7. Data analyses

Trimmed Spearman-Karber analysis was used to calculate the median lethal concentration (LC50) and median lethal residue (LR50) values using ToxCalc software (Version 5.0, Tidepool Scientific, McKinleyville, CA). For survival data, one-way analysis of variance (ANOVA) was used to determine differences between means, using a 0.05 level of significance. Survival data were transformed by arcsine square root prior to statistical analysis. Dunnett's test was used to determine significant differences from the control for concentration or residue means using ToxCalc software. BCF values (L kg^{-1}) were calculated as

$$BCF = \frac{C_{tiss}}{C_w}$$

where C_{tiss} is the mean tissue concentration (μmol kg^{-1} ww) at exposure termination, and C_w is the mean water concentration during the entire exposure period (μmol L^{-1}). For exposures to TNT and TNB, BCFs were calculated for the sum concentration of parent and transformation products, unless noted otherwise.

3. Results

3.1. Water quality

Water quality parameters were well within acceptable levels (USEPA, 2002) and were not statistically different between treatments. The dissolved oxygen ranged from 5.9 to 9.0 mg L^{-1}, temperature ranged from 22.0 to 24.1 °C, pH ranged from 7.3 to 8.3, and salinity ranged from 20 to 22 psu in all experiments.

3.2. Static-renewal TNT toxicity experiment

3.2.1. Exposure water

Formation of the TNT transformation product 4-ADNT was observed in all TNT solution treatments and its concentration was higher for target concentrations ≤ 1 mg L^{-1} (Table 1) during the 24-h period between exposure water renewals. Loss of compounds also occurred during the period following exposure to water renewal, with the mean TNT+4-ADNT water concentrations decreasing 40–69% during each 24 h period following renewal of exposure water (Table 1; Fig. 1).

3.2.2. Mortality

Significant mortality occurred in the three highest concentrations by day 10 (Table 1). No mortality occurred in the control. The LC50 values (and 95% confidence intervals) calculated using mean sum concentrations of TNT and 4-ADNT and survival data for days 4, 5, and 10 of exposure were 2.4 (2.3–2.6), 2.4 (2.2–2.6), and 2.3 (2.2–2.5) mg L^{-1}, respectively. The LC50 values were similar for days 4, 5, and 10, as most mortality occurred before day four.

3.2.3. Bioaccumulation and critical body residues

Upon termination of the 10-d exposure period, TNT and its transformation products 2-ADNT and 4-ADNT were detected in fish tissues, at mean fractions of the total body residue of 24.6–30.3% for TNT, 42.6–45.2% for 2-ADNT, and 25.4–29.7% for 4-ADNT. The mean whole-body residues are reported as the sum of TNT, 2-ADNT, and 4-ADNT (SumTNT). The SumTNT body residues increased with concentration of these compounds in the water (Table 2). The bioconcentration factors (BCFs) for SumTNT were all similar at different exposure concentrations (Table 2). The BCFs determined for TNT-only mean water and tissue concentration were 0.83, 0.67, 0.74, 0.78, and 0.66 L kg^{-1} ww, from lowest to highest treatment.

The relationship between mortality and whole-body residue was evaluated to determine critical body residues in JSHM. Body residues associated with significant mortality are indicated in Table 2. The 10-d LR50 for SumTNT, determined using day 10 mortality, was 5.4 mg kg^{-1} ww (95% confidence interval: 4.7–6.3 mg kg^{-1}), or 26.1 μmol kg^{-1} ww.

3.3. Flow-through TNT toxicity experiment

3.3.1. Exposure water

Concentration of SumTNT in the exposure chambers was observed to gradually decline during the 10-d exposure period (Fig. 1), resulting in mean percent concentration decrease ranging from 11% at the highest concentration to 20% at the lowest (Table 3). The decreasing trend was less pronounced at higher initial concentrations. Transformation of TNT was minimal throughout the experiment, with the only detectable transformation product (4-ADNT) representing $<2.4\%$ of the mean sum concentration of TNT and 4-ADNT (Table 3).

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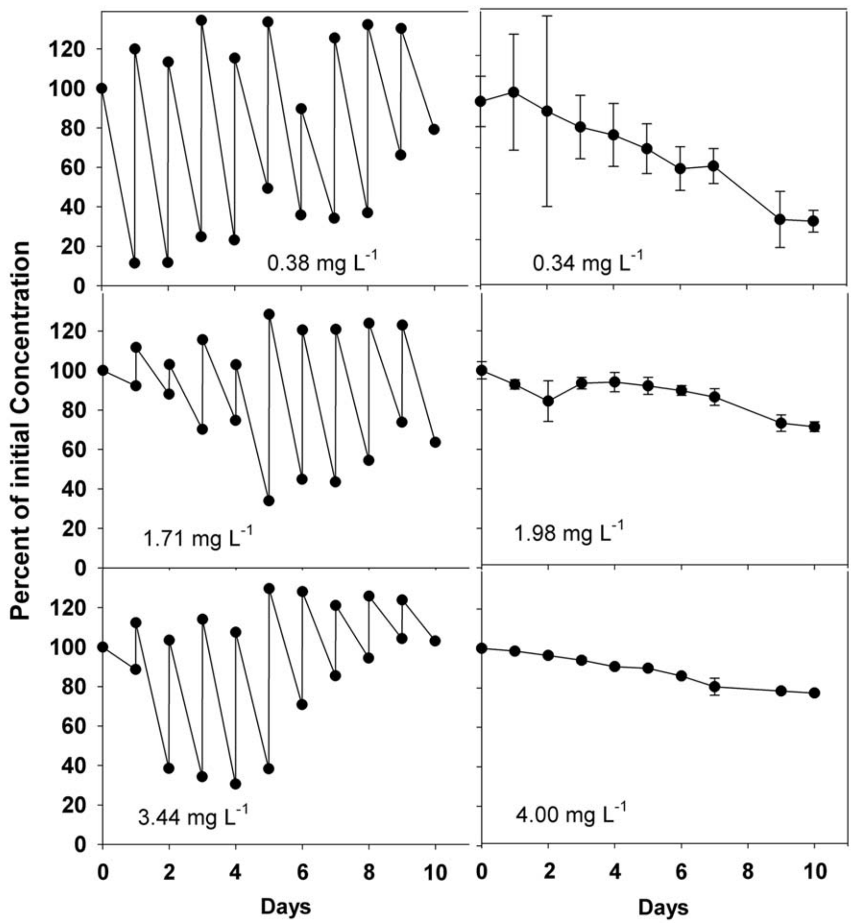

Fig. 1. Temporal change in aqueous concentrations of SumTNT during the TNT 10-d static renewal experiment (left graphs) and the TNT 10-d flow-through experiment (right graphs) expressed as percent of the initial (day 0) concentration with time for all treatments.

Table 2
Static-renewal TNT toxicity experiment. Mean measured water concentrations, body residues, bioconcentration factors (BCF), and percent of the total SumTNT body residue corresponding to TNT, 2-ADNT and 4-ADNT in juvenile sheepshead minnows exposed for 10 days. Numbers in parentheses are ±1 standard deviation. Numbers in bold indicate fish survival significantly different from the control. ND indicates not determined because adequate fish mass for chemical analysis obtained from one replicate only.

Mean water concentration (mg L^{-1})	Mean body residue		Mean BCF (L kg^{-1})	Mean % of total body residue		
	(mg kg^{-1} ww)	(μmol kg^{-1} ww)		TNT	2-ADNT	4-ADNT
0.31	0.69 (0.05)	3.34 (0.23)	2.25 (0.16)	29.4 (2.2)	45.2 (2.5)	25.4 (1.6)
0.69	1.56 (0.33)	7.61 (1.61)	2.2 (0.24)	24.6 (1.9)	45.7 (1.9)	29.7 (1.3)
1.52	3.41 (0.66)	16.66 (3.24)	2.29 (0.41)	26.6 (3.1)	44.2 (6.6)	29.3 (8.4)
2.38	**5.79 (1.20)**	**28.14 (6.12)**	2.43 (0.51)	30.3 (9.5)	42.6 (3.0)	27.1 (4.7)
3.14	**6.75 (ND)**	**32.90 (ND)**	2.15 (ND)	27.3 (ND)	43.4 (ND)	29.3 (ND)

Table 3
Flow-through TNT toxicity experiment. Initial (day 0) and mean sum concentrations of TNT and 4-ADNT, mean percent of the total concentration corresponding to 4-ADNT, and mean percent decline of the sum concentrations of TNT and 4-ADNT at termination of the 10-d exposure. Numbers in parentheses are ±1 standard deviation. Number in bold indicates fish survival significantly different from the control.

Day 0 exposure water	Days 0–10 exposure water			Mean survival (%)
Measured concentration (mg L^{-1})	Mean measured concentration (mg L^{-1})	% 4-ADNT	% decrease TNT+4-ADNT	
0	0	0	0	97.9 (4.2)
0.34	0.27 (0.07)	2.3 (2.3)	20.0 (5.3)	95.8 (4.8)
0.64	0.51 (0.10)	2.4 (1.6)	20.0 (3.8)	87.5 (8.3)
1.04	0.88 (0.15)	1.8 (1.2)	15.7 (2.7)	91.7 (11.8)
1.98	1.73 (0.18)	1.5 (0.7)	12.2 (1.3)	77.1 (12.5)
4.00	3.57 (0.33)	0.9 (0.4)	10.8 (1.0)	**29.2 (17.3)**

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3.3.2. Mortality

Mean percent survival was significantly lower in the highest treatment after 10-d (Table 3). A 10-d LC50 value of 2.5 mg L^{-1} was calculated using mean concentrations of TNT and 4-ADNT. Confidence limits were not calculated due to lack of complete mortality at the highest exposure concentration.

3.4. TNT and TNT transformation products comparative toxicity experiment

3.4.1. Exposure water

Mean water concentrations of 2-ADNT and 2,4-DANT compounds remained relatively constant during the 5-d exposure period (Table 4) and transformation products of these compounds were not detected in the water. Loss of compounds between exposure water renewals (i.e., during the 24-h period following each water exchange) was high in the TNT and TNB exposures (Table 4). Moreover, the formation of transformation products, 4-ADNT in the TNT experiment and DNAs in the TNB experiment, was also observed. Overall, compound transformation and loss decreased with increase in target concentration.

3.4.2. Mortality

Mean control survival remained higher than 90% up to exposure day 4, and declined to 75% by day 5, when whole fish were sampled for body burden evaluation. Mortality in contaminant treatments is reported as observed, without correction for control mortality. Mean mortality increased with aqueous concentration for the TNT, 2-ADNT, and TNB exposures (Fig. 2). No significant mortality was observed in the DANT experiment. Significant fish mortality was observed at similar water concentrations in the TNT and TNB exposures. The 5-d LC50 values (Table 5) were calculated using 5-d survival data and mean measured water concentrations across the 5-day experiment. An LC50 value could not be calculated for 2,4-DANT due to low toxicity. The LC50 for the TNT experiment was 5-times lower than that for 2-ADNT, 30-times lower than the highest concentration of 2,4-DANT, but was 1.4 times higher than that for TNB (Table 5).

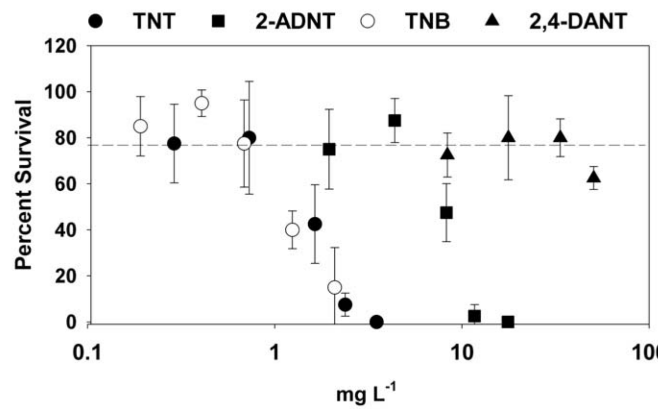

Fig. 2. Mean percent survival of juvenile sheepshead minnows at termination of the 5-d exposure to aqueous exposures to TNT, 2-ADNT, TNB and 2,4-DANT (on log scale). Error bars are ±1 standard deviation. Dashed line indicates mean control percent survival.

Table 4
Nitroaromatic compound comparative toxicity experiment. Target and mean concentrations of nitroaromatic compounds, mean percent of transformation product, and percent decrease in concentration of exposure water after the 24-h period following each water exchange. Numbers in parentheses are ±1 standard deviation.

Target concentration (mg L^{-1})	Mean measured concentration (mg L^{-1})	Mean % transformation product in 24-h aged water	Mean % 24-h decrease in total concentration
TNT exposure			
	TNT+4-ADNT	% 4-ADNT	TNT+4-ADNT
0.5	0.31 (0.10)	13.7 (4.3)	54.4 (11.5)
1.0	0.73 (0.16)	9.0 (8.9)	42.7 (21.1)
2.0	1.64 (0.29)	8.5 (8.0)	19.2 (12.6)
3.0	2.38 (0.62)	9.9 (8.2)	20.6 (18.2)
4.0	3.50 (0.38)	1.9 (0.4)	19.9 (12.0)
2-ADNT exposure			
	2-ADNT		2-ADNT
2.0	1.95 (0.10)		5.0 (5.0)
4.0	4.37 (0.17)		2.0 (0.9)
8.0	8.26 (0.20)		4.1 (6.4)
12.0	11.67 (0.45)		3.9 (5.3)
20.0	17.56 (1.58)		0.0 (0.0)
2,4-DANT exposure			
	2,4-DANT		2,4-DANT
12.5	8.35 (0.47)		6.4 (8.7)
25	17.69 (0.43)		3.4 (4.2)
50	33.53 (1.39)		2.0 (2.8)
75	50.25 (1.30)		4.0 (2.3)
TNB exposure			
	TNB+DNAs	% DNAs	TNB+DNAs
0.5	0.19 (0.04)	20.1 (2.9)	70.6 (19.2)
1.0	0.40 (0.03)	17.0 (8.9)	61.0 (11.2)
1.5	0.69 (0.03)	15.4 (6.0)	48.3 (6.9)
2.5	1.25 (0.11)	11.9 (9.5)	46.9 (12.4)
4.0	2.09 (0.40)	7.0 (5.0)	25.5 (6.5)

Table 5
Nitroaromatic compound comparative toxicity experiment. Median lethal concentrations (LC50) calculated using juvenile sheepshead minnow 5-d survival data. Numbers in parenthesis are 95% confidence intervals. ND indicates not determined because of insufficient mortality.

Compound	LC50 (95% CI) (mg L^{-1})
TNT	1.7 (1.6–1.8)
2-ADNT	8.6 (7.1–9.6)
2,4-DANT	ND; >50.3
TNB	1.2 (1.2–1.3)

3.4.3. Bioaccumulation and lethal body residues

Exposure to SumTNT, 2-ADNT, and 2,4-DANT resulted in detectable accumulation of nitroaromatic compounds in fish tissues at termination of the 5-d exposure (Table 6). Detectable levels of nitroaromatic compounds were not observed in the tissues of fish exposed to TNB. Exposure to SumTNT resulted in the bioaccumulation of TNT, 2-ADNT, and 4-ADNT in the tissues (Table 7). In the lowest treatment the mean fractions of the total body residue was 52.8% for TNT and 47.2% for 2-ADNT. In the remaining treatments, the relative contribution of compounds to the total body residue was similar: 25.6–33.3% for TNT, 24.2–35.9% for 2-ADNT, and 30.9–50.1% for 4-ADNT (Table 7). The observation that 2-ADNT but not 4-ADNT was detected in the lowest treatment is probably an artifact of the low exposure level, as the reported tissue concentrations for that treatment were lower than the laboratory reporting of 1 mg/kg. The higher exposure treatments yield more reliable data on the relative concentration of 4-ADNT and 2-ADNT. Only the target compounds were detected in tissue extracts of fish exposed to 2-ADNT and 2,4-DANT.

Total nitroaromatic compound body residues (sum concentration) increased with increase in exposure water concentrations for the TNT, 2-ADNT, and 2,4-DANT experiments (Table 6). The mean BCFs were highest for 2-ADNT, intermediate for SumTNT,

Table 6
Nitroaromatic compound comparative toxicity experiment. Mean measured water concentrations, body residues, and bioconcentration factors (BCF) in juvenile sheepshead minnows. Numbers in parentheses are 1 standard deviation. Numbers in bold indicate concentration associated with mean fish survival significantly decreased relative to the control. ND indicates parameter not determined because of lack of or insufficient surviving fish.

Mean water concentration ($mg\ L^{-1}$)	Mean body residue		Mean BCF ($L\ kg^{-1}$)
	$mg\ kg^{-1}$ ww	$\mu mol\ kg^{-1}$ ww	
TNT Exposure			
0.31 (0.10)	0.59 (0.20)	2.61 (0.89)	1.82 (0.65)
0.73 (0.16)	2.64 (1.33)	11.65 (5.86)	3.33 (1.79)
1.64 (0.29)	**4.35 (0.35)**	**19.15 (1.54)**	2.39 (0.18)
2.38 (0.62)	**5.77 (ND)**	**24.84 (ND)**	2.24 (ND)
3.50 (0.38)			
2-ADNT exposure			
1.95 (0.10)	12.84 (0.53)	65.19 (2.69)	8.00 (3.30)
4.37 (0.17)	28.00 (0.91)	142.14 (4.64)	5.61 (1.41)
8.26 (0.20)	**52.70 (3.19)**	**267.52 (16.20)**	6.38 (0.39)
11.67 (0.45)	ND	ND	ND
17.56 (1.58)	ND	ND	ND
2,4-DANT exposure			
8.35 (0.47)	4.79 (1.18)	28.68 (7.06)	0.57 (0.14)
17.69 (0.43)	14.02 (0.76)	83.96 (4.54)	0.79 (0.04)
33.53 (1.39)	31.52 (6.19)	188.76 (37.08)	0.94 (0.18)
50.25 (1.30)	28.22 (5.22)	151.64 (31.26)	0.94 (0.10)

Table 7
Nitroaromatic compound comparative toxicity experiment**.** Percent of the total SumTNT body residue corresponding to TNT, 2-ADNT, and 4-ADNT in juvenile sheepshead minnows exposed to TNT. Numbers in parentheses are ± 1 standard deviation. ND indicates parameter not determined because of lack of or insufficient surviving fish.

Mean water concentration ($mg\ L^{-1}$)	% of body residue		
	TNT	2-ADNT	4-ADNT
0.31	52.8 (10.3)	47.2 (10.3)	0.0 (ND)
0.73	33.2 (19.0)	35.9 (27.5)	30.9 (23.6)
1.64	25.6 (9.8)	24.2 (1.2)	50.1 (9.5)
2.38	33.3 (ND)	29.8 (ND)	39.3 (ND)
3.50	ND	ND	ND

and lowest for 2,4-DANT (Table 6). The BCF values determined for TNT (parent compound only) using mean water and mean tissue concentration were 1.26, 1.76, 0.76, and 1.19 $L\ kg^{-1}$, from lowest to highest treatments. Those values were lower than those determined for SumTNT (Table 6).

Mean body residues associated with treatments for which significant mortality was observed are reported in Table 6. Lethal body residues, expressed as 5-d LR50 value, was 4.63 $mg\ kg^{-1}$ ww (95% confidence interval: 3.52–4.8 $mg\ kg^{-1}$ ww), or 20.4 $\mu mol\ kg^{-1}$ ww for SumTNT. The 5-d LR50 for 2-ADNT was 54.2 $mg\ kg^{-1}$ ww (275.3 $\mu mol\ kg^{-1}$ ww). Confidence intervals could not be calculated because body residues in the highest 2-ADNT treatment with surviving fish was not determined due to insufficient tissue mass and therefore not included in the calculation.

4. Discussion

4.1. Exposure water

Exposure water was exchanged approximately every 24 h during the static-renewal experiments. In the TNT 5-d and 10-d static renewal exposures, however, formation of 4-ADNT during the 24 h period between water renewal occurred in all treatments. In addition, the concentration of SumTNT in the beakers decreased by 40% or more during that period, suggesting that loss as transformation to undetected compounds likely occurred. Transformation of TNT to aminated transformation products during static or static-renewal exposures has been reported previously (Carr and Nipper, 2000; Nipper et al., 2001; Conder et al., 2004a; Ownby et al., 2005; Yoo et al., 2006; Rosen and Lotufo, 2007a). The decline of SumTNT concentrations between water exchanges observed in similar static-renewal toxicity tests with fathead minnows (Yoo et al., 2006) was of a similar magnitude as that observed in similar experiments reported in the present study. Feeding experimental fish may have contributed to the observed high loss of TNT during the experiment, as the presence of non-ingested food and egested material has been shown to enhance microbial activity responsible for the transformation of TNT (Yoo et al., 2006). In aqueous exposures to TNT under conditions similar to the present study, but without addition of food, transformation was appreciably less pronounced (Conder et al., 2004a; Belden et al., 2005; Lotufo and Lydy, 2005; Rosen and Lotufo, 2007a). The only TNT transformation product detected in the exposure water was 4-ADNT, contrasting with previous reports of the formation of both congeners during aqueous exposures (Carr and Nipper, 2000; Ownby et al., 2005; Yoo et al., 2006; Rosen and Lotufo, 2007a). Preferential biotransformation of TNT to 4-ADNT has been previously observed in soils and sediments (Conder et al., 2004b; Lachance et al., 2004) and has been characterized as thermodynamically preferable (McCormick et al., 1976), at least partially explaining the absence of 2-ADNT as a transformation product of TNT in the exposure water.

Reductive transformation of TNB, leading to the formation of dinitroanilines or amino-dinitrobenzenes, was more extensive than that of TNT in this study. Substantial transformation of TNB has also been observed in spiked sediments (Steevens et al., 2002; Lotufo and Farrar, 2005) over 24 h, which is surprising since TNB is considered environmentally persistent, not readily biodegradable, and has a high propensity to contaminate groundwater near production waste disposal sites (Reddy et al., 1997).

The concentration of 2-ADNT and 2,4-DANT did not change appreciably between water exchanges; therefore transformation or loss of these compounds was negligible. Persistence of ADNTs and DANTs in experimental systems has been reported previously for sediment (Lotufo and Farrar, 2005), soils (Lachance et al., 2004), and water (Conder et al., 2004a).

Unlike the static-renewal experiments, the TNT exposure conducted using a diluter board delivered exposure water every hour and was expected to maintain stable concentrations of TNT. However, the exposure water concentration did decline gradually during the course of the 10-d experiment. The presence of non-ingested food and egestion detritus in the chambers likely promoted transformation of the TNT delivered to the exposure chambers. To compensate for this incremental loss, a reciprocal incremental increase of the TNT concentration in the water delivered to the chamber would have been necessary to attain stable exposure concentrations.

4.2. TNT toxicity

The lethal toxicity of TNT to JSHM determined using two different exposures types yielded similar 10-d LC50 values expressed as the sum concentration of TNT and transformation products. The 5-d LC50 determined in the comparative toxicity experiment was lower than the 10-d LC50s obtained from the

Table 8
Median lethal concentrations (96-h LC50) reported for fish exposed to TNT.

Species	96-h LC50 (mg L^{-1})	Reference
Cyprinodon variegatus	1.7	This study
Pimephales promelas	2.2–3.7[a]	Liu et al. (1983a,b), Pearson et al. (1979), and Yoo et al. (2006)
Lepomis macrochirus	2.6–3.4[a]	Nay et al. (1974) and Liu et al. (1983a)
Oncorhynchus mykiss	0.8–2.0[a]	Liu et al. (1983a)
Ictalurus punctatus	2.4–3.3[a]	Liu et al. (1983a)

[a] Lowest and highest reported values.

Table 9
Median lethal concentrations (LC50) reported for fish and aquatic invertebrates exposed to TNT, ADNTs, DANTs, and TNB.

Species	LC50 (mg L^{-1})					
	TNT	2-ADNT	4-ADNT	2,4-DANT	2,6-DANT	TNB
Cyprinodon variegatus	1.7	8.6	ND	> 50.3[f]		1.2
Pimephales promelas[a]	2.4	14.8	6.9			1.0
Daphnia magna[a]	11.9	4.5	5.2			2.7
Daphnia magna[b]	5.1	1.1	5.1			
Ceriodaphnia dubia[c]	> 6.0[g]	4.9	6.6	0.2	2.0	0.8
Hyalella azteca[d]	3.6	3.8	9.2	1.7		2.3
Chironomus tentans[e]	1.9	3.3		33.3		2.2

[a] Pearson et al. (1979).
[b] Johnson et al. (1994).
[c] Griest et al. (1998).
[d] Sims and Steevens (2008).
[e] Lotufo, unpublished.
[f] NOEC=50.3 mg L^{-1}, highest concentration used.
[g] NOEC=3.0; LOEC=6.0 mg L^{-1}.

10-d static-renewal exposure, likely because of the unintended contribution of unknown non-contaminant related stress suffered by experimental organisms, as evidenced by a decline in control survival by day 4 of the exposure. Therefore, the 5-day LC50 values reported in this study for TNT transformation products may be lower than those that would have been derived in the absence of non-contaminant stressors. The exposure chamber used in the 5-d exposures was smaller than the chambers used in the other exposures in this study, but larger than the recommended 250-ml minimum volume test vessels (USEPA 2002). Therefore, chamber size was unlikely a factor contributing to the elevated control mortality in the 5-d exposures.

The LC50 values derived in this study for JSHM are comparable to those previously determined for freshwater fish (Table 8). Exposure of the marine species *Sciaenops ocellatus* (redfish) to TNT during the hatching period resulted in a 48-h LC50 value of 8.2 mg L^{-1} (Nipper et al., 2001). While four-day toxicity data are not available for that species, the comparison of LC50s suggests a relatively higher tolerance of marine redfish compared to freshwater species.

The relative toxicity of TNT, TNB, and two major TNT transformation products, 2-ADNT and 2,4-DANT, to JSHM was investigated in this study. The relative toxicities of TNT, TNB, and two major TNT transformation products, 2-ADNT and 2,4-DANT, to sheepshead minnows can be compared using LC50 values. Nitroreduction appears to decrease the toxicity of TNT to JSHM, as the LC50 for the mono-aminated compound 2-ADNT was approximately 5 times as high as that for TNT and further amination decreases toxicity even more dramatically, as the highest tested concentration of the more reduced compound 2,4-DANT was 30 times higher than the TNT LC50 (Table 5). The toxicity of 2-ADNT was also less than that of TNT for the fathead minnow (Table 9), but for invertebrates, 2-ADNT exhibited lesser toxicity compared to TNT to invertebrate species, except for daphnids (Table 9). While not lethal to the sheepshead minnow at the concentrations used in this study, 2,4-DANT was 17 times less toxic than TNT for the midge *Chironomus tentans*, but was more toxic than TNT to an amphipod *Hyalella azteca* (2 times) and the cladoceran *Ceriodaphia dubia* (30 times) (Griest et al., 1998). Substantial differences between the lethal toxicity of ADNT and DANT isomers were observed for fish and invertebrate species (Table 9), but were not investigated here for JSHM. Compared to TNT, TNB was more toxic to both JSHM and fathead minnow, as well as for most invertebrates investigated (Table 9).

4.3. Biotransformation and bioconcentration

Exposure of fish to TNT, 2-ADNT, and 2,4-DANT resulted in accumulation of nitroaromatic compounds in their tissues at termination of the 5-d exposure. Fish exposed to TNB, however, did not accumulate detectable amounts of TNB in their tissues. Reductive transformation of TNB in the exposure water resulted in significant transformation of that compound to DNAs, which were not detected in the tissues.

For TNT exposures, the transformation products 2- and 4-ADNT, in addition to TNT parent compound, were present in the tissues of exposed fish. The compound 4-ADNT present in the tissue could be due to biotransformation of TNT within the fish, uptake from the exposure water, or both. The relative contribution of each source can be estimated using the BCF value of 8 L kg^{-1} derived for the lowest 2-ADNT exposure (Table 6) and the estimated concentration of 4-ADNT in the exposure water. For the 5-d exposure to 0.73 mg L^{-1} TNT treatment, the mean 4-ADNT body residue was 0.82 mg kg^{-1} and the expected body residue from direct uptake, estimated using the above BCF value, was 0.38 mg kg^{-1}, or 46% of the total. Therefore, based on this result and a toxicokinetics study (Lotufo and Lydy, 2005), the metabolic formation of 4-ADNT in JSHM provided a significant pathway for the bioaccumulation of 4-ADNT in JSHM in the 5-d experiments. Because the compound 2-ADNT was not detected in the exposure water, it was assumed as derived from the biotransformation of TNT taken up by the fish. The process of biotransformation of TNT to aminated transformation products has been reported in the scientific literature for aquatic fish (Ownby et al., 2005; Lotufo and Lydy, 2005) and invertebrates (Conder et al., 2004a; Rosen and Lotufo, 2005), as well as in terrestrial invertebrates (Renoux et al., 2000; Dodard et al., 2004). The body concentrations of the ADNTs were much higher than TNT in catfish, even for TNT exposure periods as short as one hour (Ownby et al., 2005).

The BCF of a compound is the most frequently used indicator of its tendency to concentrate in aquatic organisms in aqueous exposures (Meylan et al., 1999). Predictive models (e.g., Veith et al., 1979; Meylan et al., 1999) that estimate BCF values for

organic compounds based on their hydrophobicity as represented by their n-octanol/water partitioning coefficient (K_{ow}), reveal that the nitroaromatic compounds investigated in this study are expected to accumulate in the tissues at concentrations, less than one order of magnitude higher than the exposure water concentrations (Lotufo et al., 2009). The BCF values determined in this study were similar to values predicted using the equation derived by Veith et al. (1979) and presented in Lotufo and Lydy (2005) for 2-ADNT (9.1 L kg^{-1}), 2,4-DANT (0.5 L kg^{-1}). However, the experimental BCFs in this study were lower than the similarly predicted value for TNT (4.6 L kg^{-1}). The lower TNT BCFs determined in the present study relative to values determined in a 6-h kinetics exposure (9.6 L kg^{-1}) (Lotufo and Lydy 2005) may be explained by the longer exposure duration. The longer exposure duration likely allowed transformation and elimination to become more efficient, thereby resulting in lower net bioaccumulation. The TNT BCF determined for juvenile catfish (0.8 L kg^{-1}) (Ownby et al., 2005) was lower than the BCFs for fathead and sheepshead minnows, as expected due to the apparent higher biotransformation capability reported for the catfish (indicated by the higher ratio of biotransformation products to parent compound in catfish). Reported TNT BCFs for aquatic invertebrates range from 0.8 to 4.0 L kg^{-1} (Belden et al., 2005; Conder et al., 2004a; Rosen and Lotufo, 2007b), likely as a result of different exposure conditions as well as biotransformation capabilities among organisms. The BCFs obtained in this study for 2-ADNT (6.4–8 L kg^{-1}) were higher while those for 2,4-DANT were higher than the BCF (13.1 and 0.5 L kg^{-1}, respectively) derived from toxicokinetics data for sheepshead minnow (Lotufo and Lydy 2005).

4.4. Critical body residues

Critical body residues (CBRs) are reported in this study as the whole body concentration that was associated with significant mortality of JSHM upon termination of the exposure period. The CBRs for SumTNT (19.15–32.9 μmol kg^{-1} ww), represented by the sum of TNT and ADNTs, were similar to the 10-d CBR (15.3 μmol kg^{-1} ww) determined for juvenile fathead minnows (Yoo et al., 2006). The CBRs for TNT, expressed as median lethal residue (LR50), were 172 μmol kg^{-1} ww for the aquatic oligochaete *Tubifex tubifex*, 118.0 μmol kg^{-1} ww for the midge *Chironomus tentans*, and 11.7–39.4 μmol kg^{-1} ww for the amphipod *Eohaustorius estuarius* (Conder et al., 2004c; Rosen and Lotufo, 2005), suggesting relatively constant critical body residues for aquatic animals. The range of CBRs for TNT is substantially lower than the range considered typical for compounds causing mortality by general narcosis, meaning that lethal effects of TNT are likely associated with specific modes of action.

5. Conclusions

The observed low toxicity and bioconcentration potential of TNT are consistent with previously reported values for other aquatic animals, with reductive transformation of TNT to 2-ADNT and 2,4-DANT in the environment expected to result in decreased toxicity to fish. Measured bioconcentration values for those compounds corroborated the low bioaccumulation potential predicted by their low hydrophobicity. Concentrations of TNT and its major transformation products expected to promote adverse effects to fish populations in the marine environments are likely orders of magnitude higher than concentrations (part-per-billion or less) expected in areas where exposed UXO are present.

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